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Spontaneous Formation of Fibrillar β-Sheet Assemblies from Peptide-Grafted Polyamine; Effect of Complexation with Poly(ethylene glycol) Derivatives

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Amphiphilic graft copolymer, polyallylamine bearing poly(γ-methyl-L-glutamate) graft chains (PAAgPMLG), was prepared. The conformation of PAAgPMLG was changed spontaneously from α-helix to β-sheet only when the amino groups of PAA units were protonated (pH < 8). In this condition, PAAgPMLG formed amyloid-like fibrils with regular quaternary structure. On the other hand, such fibril formation with α-to-β structural transition was obviously inhibited in the presence of carboxylic acid-terminated poly(ethylene glycol), which prevents appropriate folding of peptide graft-chain through the complexation with amino groups of PAAgPMLG. These findings demonstrate that the amyloid fibril formation seems to occur if peptide chains are folded appropriately even at the simple synthetic peptide, which have no specific protein sequences.

Key words: Polyglutamate, Amyloid Fibril, β-Sheet Structure, Self-Assembly, Poly(ethylene glycol)

1. INTRODUCTION

The conformations of peptides and proteins play a key role in defining their functions. Recently, much efforts have been directed toward the design and synthesis of model protein to elucidate interactions involved in protein folding, and to develop protein-based materials.[1] In particular, the self-assembly of peptides and proteins into β-sheet-rich high ordered fibrillar structure has attracted the attention[2] because of the characteristic structure of these assemblies and because of their association with neurodegenerative diseases like Alzheimer's disease and Creutzfeldt-Jacob's disease.[3] So it is important to construct peptide β-sheet assemblies and elucidate their molecular-level structure to understand the pathogenesis and therapeutics of these diseases. In previous studies,[4] several proteins have been identified in amyloid aggregates, and these aggregates were found to have a common core structure. The main feature is the core structure in which continuous β-sheets lie parallel to the long axis of a fiber, while their constituent β-strands run perpendicular to this axis. Recently, Dobson's group found that two non-pathogenic proteins also form amyloid fibrils in vitro under particular conditions and suggested that the formation of amyloid fibrils is a property common to many of the proteins.[5] This possibility can readily be supported by the following facts; (a) amyloids have a common core structure even though the proteins involve no obvious sequential or structural similarities, (b) the intermolecular hydrogen bonds that stabilize this architecture involve the peptide backbone, which is common to all proteins. If the formation of amyloid fibril is a truly genetic property of the natural peptide, it should be possible to generate such fibrils even at the simple synthetic peptide, which have no specific protein sequences, if appropriate conditions could be found.

In this paper, we describe the preparation of an amphiphilic artificial protein with an α-helical homopolypeptide, poly(γ-methyl-L-glutamate)-grafted polyallylamine (PAAgPMLG), and their conformational characteristics in aqueous solution. In addition, effects of environmental factors, *i.e.*, pH of the solution or molecular interaction between PAAgPMLG and poly(ethylene glycol) derivatives, on the self-assembling properties of PAAgPMLG were examined.

2. EXPERIMNTAL SECTION

2-1. Materials.

The poly(γ-methyl-L-glutamate) grafted polyallylamine (Fig. 1) was prepared as follows. First, PAA (M_w = 10000) (1.0 g) was dissolved in 10 mL of water (pH 9.5), and then the dioxane solution (10 mL) of di-*tert.*-butyl dicarbo-nate (0.96 g) was added slowly. After stirring for 8 h at room temperature, 50 mL of NaOH solution (1M) was added into the reaction mixture and the precipitate was washed with water and lyophilized. As a result, the PAA copolymer whose amino groups (40%) were protected with BOC groups ($BOCPAA_{0.4}$-*co*-$PAA_{0.6}$) was obtained (1.42 g). The ratio of the free amino groups to the BOC-protected amino groups was estimated by means of ^{1}H-NMR spectroscopy. Next, graft-polymerization of γ-methyl-L-glutamate-*N*-carboxy anhydride (MLG-NCA) was carried out from free amino groups of the BOCPAA-*co*-PAA in chloroform (150 mL) for 40 h at room temperature. Then the reaction

mixture was poured into a large excess of diethyl ether, and the precipitate was washed with diethyl ether and 1,2-dichloroethane repeatedly (0.80 g). The chemical structure of BOCPAAgPMLG was confirmed by IR, ^{1}H-NMR and elemental analyses, and the number-average degree of polymerization (n) of the PMLG graft chain was estimated to be 14. Finally, the desired PMLG grafted PAA (PAAgPMLG) was obtained by removal of the BOC groups in trifluoroacetic acid solution (0.62 g). The α-methoxy-ω-carboxyl-poly(ethylene glycol) (PEG-COOH Av. M_w;5000) and the α-methoxy-ω-amino-poly-(ethylene glycol) (PEG-NH_2 Av. M_w;5000) were purchased from Sigma Chemical Co. and Funakoshi Co., Ltd., respectively, and used without further purification.

2-2. Circular Dichroism (CD) and Fourier Transform Infrared (FTIR) Spectroscopy Measurements.

CD spectra were recorded on a J-720 WI spectropolarimeter (JASCO Ltd.) under a nitrogen atmosphere. The pH of the sample solution was adjusted with 0.1 M HCl or 0.1 M NaOH. Experiments were performed in a quartz cell with 5 mm path length, over the range 190-250 nm, at ambient temperature. Sample solutions were prepared by diluting the 2,2,2-trifluoroethanol (TFE) stock solution of PAAgPMLG with purified water (Final peptide concentration was 1.1 x 10^{-4} glutamate unit M, TFE content 20%). The helix content was calculated by using curve fitting method (Greenfield et al. 1969).[6] Transmission-FTIR spectra were measured by Parkin-Elmer Spectrum 2000, using a mercury-cadmium-tellurium (MCT) detector (resolution, 2 cm^{-1}; number of scans, 1024). The sample and the detector chamber were purged with dried nitrogen before and during measurement.

2-3. Atomic Force Microscopy (AFM) Measurement.

The AFM images were collected at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc.) operating in a tapping mode using silicon cantilevers (125 μm, tip radius 5-10 nm). An aliquot of PAAg-PMLG in water/TFE (8/2 v/v, pH 4.0) was placed on freshly cleaved mica. After adsorption for 3 min, the excess solution was removed by absorption onto filter paper. A 10 μm x 10 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz and the original images were sampled at the resolution of 512 x 512 points.

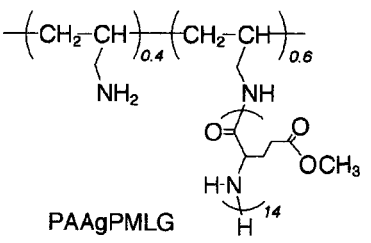

Fig. 1. Chemical structure of poly(γ-methyl-L-glutamate) grafted polyallylamine (PAAgPMLG).

3. RESULT AND DISCUSSION

3-1. Conformational Study of Polyglutamate-Grafted Polyamine at Various pHs.

The secondary structure of PAAgPMLG was investigated by means of CD and FTIR spectroscopies. Fig. 2A displays the time dependence of CD spectra for PAAgPMLG in water/TFE (8/2 v/v) at pH 4.0. It has been well known that the CD spectrum of α-helical peptide exhibits the double maximums at 222 and 208 nm, whereas the spectrum of β-sheet one exhibits the single maximum at *ca.* 217-218 nm.[6] In our case, a negative maximums at 222 and 208 nm were observed shortly after preparation of the sample solution, indicating the existence of a right-handed α-helix conformation (Fig. 2A). The helicity of the PMLG units was calculated to be about 60 % from curve fitting method.[6] This helicity is reasonable if we take into account the relatively short segment length of n = 14. On the other hand, the CD spectrum obtained after incubation for 30 h exhibited a single negative maximum at 218 nm and a positive maximum at 197 nm. This means that a conformation of PAAgPMLG was

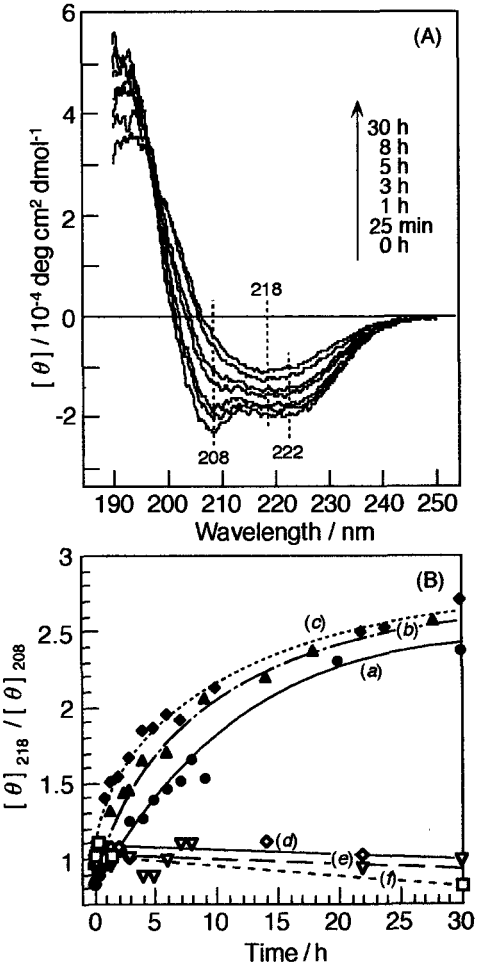

Fig. 2. (A) CD spectral changes of PAAgPMLG in water/TFE (8/2 v/v) at pH 4.0. The PAAgPMLG was incubated for the indicated periods (0-30 h) at room temperature. [MLG] = 1.1 x 10^{-4} unit M. (B) Time dependence of the ratio of molar ellipticities at 218 nm and 208 nm ($[\theta]_{218}/[\theta]_{208}$) at pH 4.0 (*a*), 4.6 (*b*), 5.4 (*c*), 8.7 (*d*), 9.2 (*e*), 9.7 (*f*).

changed spontaneously from α-helix to β-sheet. It seems that amphiphilic property of PAAgPMLG causes tight packing of hydrophobic PMLG chain in acidic condition and then the intramolecular hydrogen bonding is likely to be rearranged to the intermolecular one, which induced the conformational change of PMLG chain. Subsequently, effects of pH of the solution on the secondary structure of PAAgPMLG were examined. Fig. 2B plots the ratio of molar ellipticities at 218 nm and 208 nm ($[\theta]_{218}/[\theta]_{208}$) at various pHs as a function of incubation time. It can be seen from the figure that the time-course of $[\theta]_{218}/[\theta]_{208}$ is strongly dependent on the pH of the solution; that is, the values of $[\theta]_{218}/[\theta]_{208}$ increase with the elapse of time at pH < 8 due to an α-to-β structural transition, whereas significant change of $[\theta]_{218}/[\theta]_{208}$ is not observed at pH > 8. These results suggest that the structural transition of PAAgPMLG from α-helix to β-sheet was occurred only at pH < 8. To obtain more quantitative information on the secondary structure, transmission FTIR spectra were measured. PAAgPMLG was adsorbed onto a CaF_2 plate after incubation for 30 h in water/TFE (8/2, v/v) at pH 4.0 and 9.2, respectively. In the amide I region,[7] characteristic absorptions with the antiparallel β-sheet structure were observed at 1695 cm^{-1} and 1627 cm^{-1} in the case of pH 4.0, whereas PAAgPMLG took mainly the α-helix structure (peak maximum at 1656 cm^{-1} and shoulder at 1627 cm^{-1}) at pH 9.2. The β-sheet contents were evaluated to be 77 and 34 % at pH 4.0 and 9.2, respectively, by peak resolution of the spectra obtained. It is clear from these spectral data that the pH of the solution was responsible for regulating the self-conformational change of PMLG graft chains. The acid dissociation constant (pK_a) value of allylamine moiety was estimated to be 8.0 from potentiometric analysis.[8] On the other hand, the peptide chains involved have no pH-responsible residues. Therefore, it can be concluded that the protonation of amino groups affected the three-dimensional packing arrangement of hydrophobic PMLG chains; as a result, the structural transition of PAAgPMLG was observed only at pH < 8.

3-2. Formation of Fibrillar β-Sheet Self-Assembly from Polyglutamate-Grafted Polyamine.

The morphological changes of PAAgPMLG with an α-to-β structural transition were observed directly by AFM technique. Fig. 3 shows the time dependence of AFM images (3 x 3 μm^2) for PAAgPMLG at pH 4.0. An AFM image obtained just after the sample solution was prepared (0 h), in which the PMLG graft chain took mainly the α-helical form, revealed the presence of only nonfibrillar, globular aggregates (Fig. 3A). These globular species are probably micellar structure consisting of a hydrophobic PMLG core and a shell of protonated-allylamine unit. The average height of the globular species was determined to be 6.0 ± 1.0 nm, and the width was typically 40–70 nm. It is important to note that AFM provides accurate measurements of a sample's height above the mica substrate, but the well-known convolution of the scanning tip leads to an overestimation of the sample width.[9] On the other hand, after incubation for 48 h, the major portion of globular

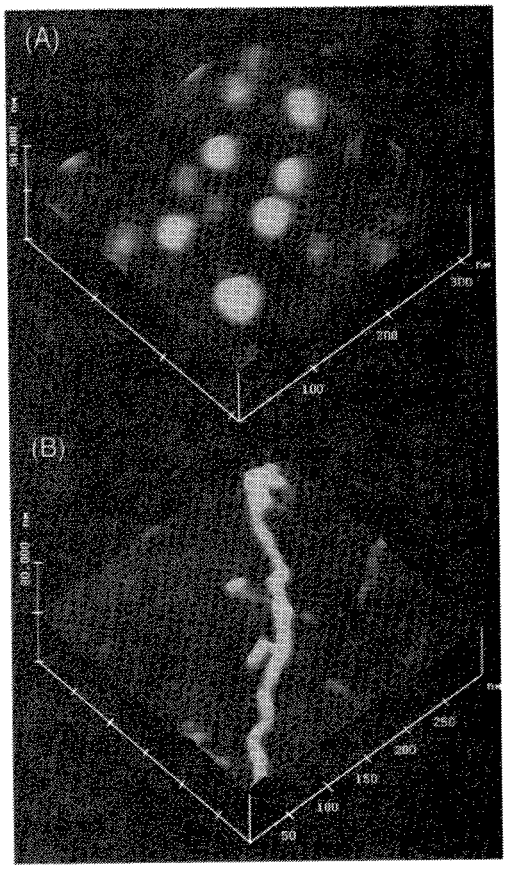

Fig. 3. Time dependence of tapping mode AFM images (300 nm x 300 nm) for PAAgPMLG. The PAAgPMLG was incubated for 0 h (A) and 48 h (B) in water/TFE (8/2, pH 4.0) at room temperature. [MLG] = 1.1 x 10^{-4} unit M.

species of PAAgPMLGs was changed into the amyloid-like fibril structures (Fig. 3B). The average height of the fibrils was determined to be 4.0 ± 1.0 nm and the length was 0.3–1.0 μm. These dimensions were in fair agreement with those of typical amyloids.[3] In addition, when the Congo Red binding studies of these fibrillar assemblies of PAAgPMLG were examined, the stained peptide assemblies exhibited a yellow birefringence under cross-polarized light. Although the birefringence differs somewhat from the green birefringence of amyloid, it is a clear indication of their anisotropy and therefore indicates the presence of a regular quaternary structure. On the other hand, this amyloid-like structure was not observed for PAAgPMLG at pH 9.2, *i.e.* at a pH at which no structural transition of PMLG graft chain was observed. Therefore, such fibril formation of PAAgPMLG was supposed to occur through the conformational transition from α-helix to β-sheet as did the other amyloidgenic proteins.

3-3. Effect of Complexation with Poly(ethylene glycol) Derivatives on Self-Assembling Properties of Polyglutamate-Grafted Polyamine.

Effect of complexation between PAAgPMLG and poly(ethylene glycol) (PEG) derivatives on conformation of PAAgPMLG was investigated by means of CD spectroscopy. PEG is important biocompatible polymer

because of their nontoxicity and nonantigenic activity. In addition, PEG derivatives are known to stabilize or preserve the three-dimensional structure of proteins and in some cases even induce helical structures to random chain polypeptides through the hydrophobic interaction.[10] Therefore, if the formation of amyloid fibril with an α-to-β structural transition is regulated truly by molecular packing of hydrophobic PMLG chains as described above, the conformation of PAAgPMLG would be affected by complexation with PEG derivatives, which prevents appropriate folding of peptide graft chain. We used two-types of PEG derivatives, that is, carboxylic acid-terminated PEG (PEG-COOH) or amine-terminated PEG (PEG-NH_2). Fig. 4 shows the time dependences of helicity of PAAgPMLG in the absence and the presence of PEG derivatives (water/TFE 8/2 v/v). From Fig. 4, the decrease in helicity of PAAgPMLG with the elapse of time was observed in the case of pH 4.0 without PEG derivatives due to an α-to-β structural transition (Fig. 4*a*). On the other hand, in the presence of PEG-COOH (Fig. 4*b-d*), such a structural transition was obviously inhibited, and, in particular, in the case of pH 6.8 (Fig. 4*d*), α-helix structure was maintained at about 60 % content even after incubation for 24 h. In this case, PAAgPMLG did not form amyloid-like fibril structure.

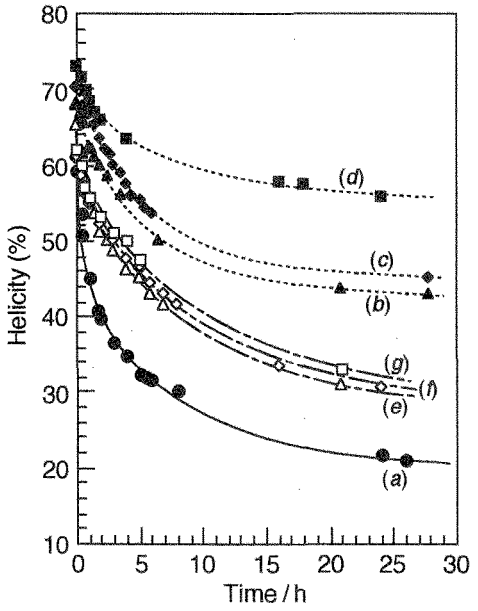

Fig. 4. Time dependences of helicity of PAAgPMLG in water/TFE (8/2 v/v) under the following conditions; (*a*) at pH 4.0 without PEG derivatives, (*b*) at pH 4.0 with PEG-COOH, (*c*) at pH 5.4 with PEG-COOH, (*d*) at pH 6.8 with PEG-COOH, (*e*) at pH 4.0 with PEG-NH_2, (*f*) at pH 5.4 with PEG-NH_2, (*g*) at pH 6.8 with PEG-NH_2. [MLG] = 1.1 x 10^{-4} unit M. [PEG derivatives]:[amine unit of PAAgPMLG] = 1:1.

Therefore, it can be assumed that the PEG-COOH interact with cationic amino-groups of PAAgPMLG through the ion-complex; as a result, the tight packing of hydrophobic PMLG chains, which induce structural change into β-sheet structure, is prevented. In fact, in the case of PEG-NH_2, which have no anionic groups, the pronounced inhibition of an α-to-β structural transition of PAAgPMLG was not observed. Thus, the conformation and morphology of PAAgPMLG could be controlled by complexation with PEG derivatives, which induced packing arrangement changes of peptide chains.

4. CONCLUSION

We successfully synthesized an artificial protein model, polyglutamate-grafted polyallylamine. Only at pH < 8, in which amino groups of PAA units were protonated, did PAAgPMLG self-convert from a globular coil into an amyloid-like fibril structure with an α-to-β structural transition. In addition, such structural transition of PAAgPMLG could be controlled by complexation with PEG derivatives, which prevent the tight packing of PMLG graft chains. These findings indicate that the three-dimensional packing arrangement of peptide chains is one of the important factors in causing fibril formation. We believe that this kind of work should be helpful in studies of the mechanisms underlying the misfolding of proteins and amyloid fibril formation.

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